Supplementary Figures

Negative regulation of IL-17-mediated signaling and inflammation by ubiquitin-specific protease 25

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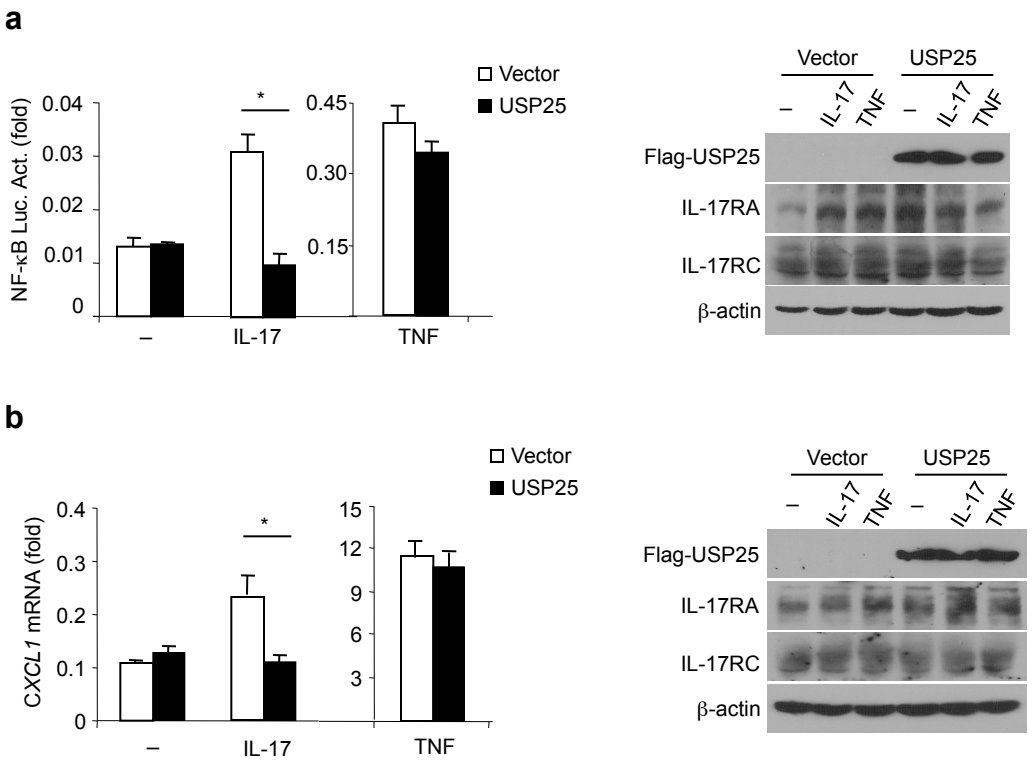
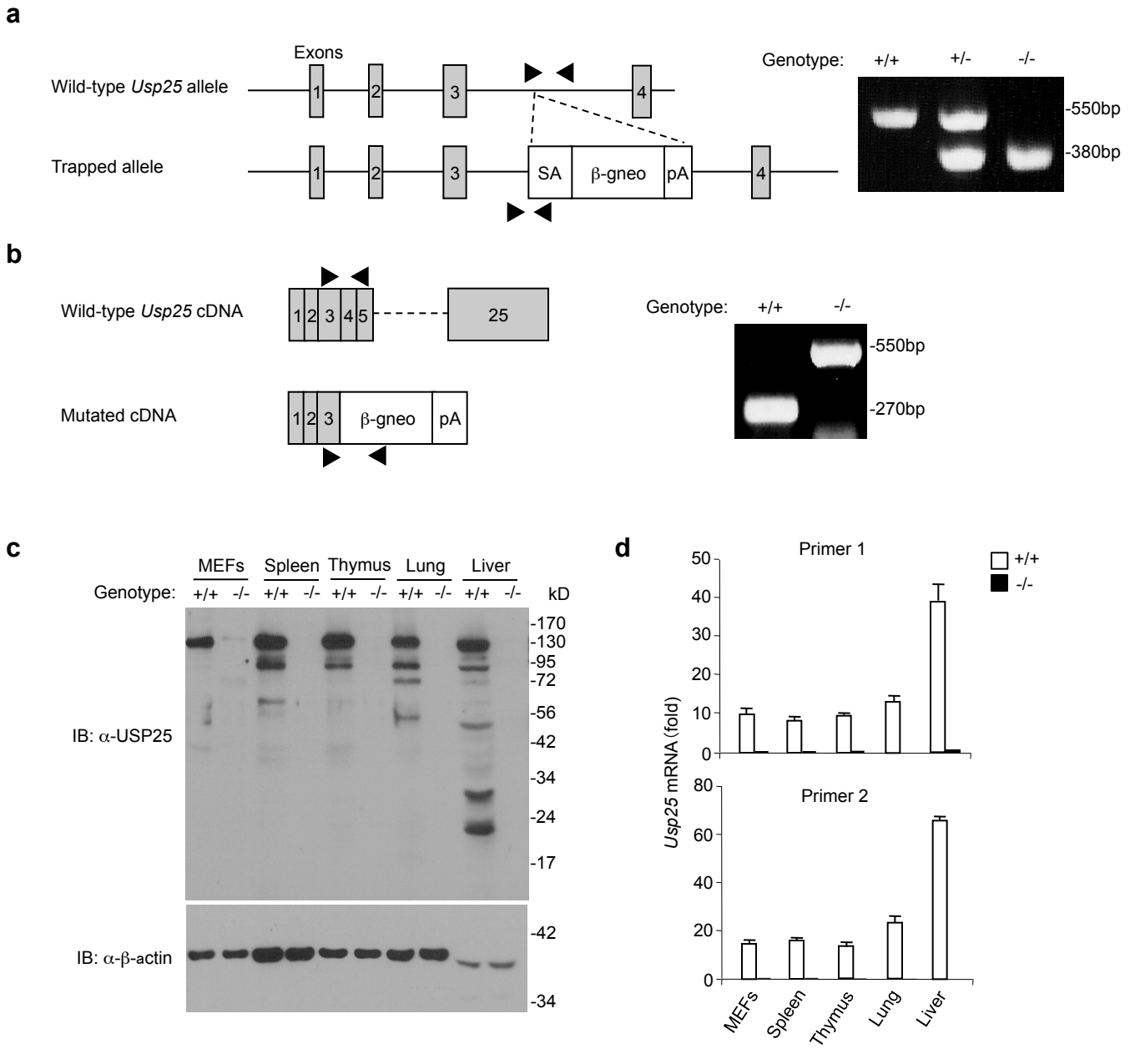
Zhong et. al., Supplementary Figure 1



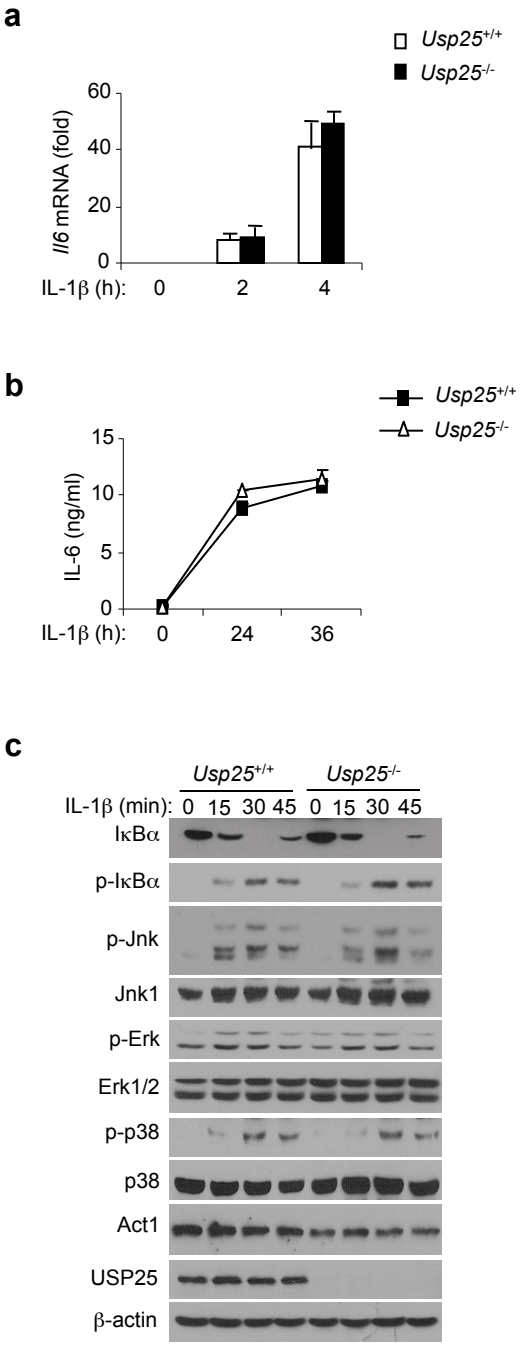
Supplementary Figure 1. Overexpression of USP25 inhibits IL-17-triggered signaling in 293T-IL-17RA/C cells. (**a**) Luciferase activity in 293T cells transfected with IL-17RA and IL-17RC (293T-IL-17RA/C) together with NF-κB reporter plasmids (0.05 μg), pRL-TK Renilla luciferase (0.01 μg) and Flag-USP25 or empty vector (Vector), followed by stimulation with IL-17 (50 ng/ml) or TNF (10 ng/ml) for 8 hours or no stimulation (-) 20 h after transfection. (**b**) Real-time RT-PCR analysis of IL-17-indued expression of *CXCL1* in 293T-IL-17RA/C cells transfected with Flag-USP25 or empty vector (Vector) and stimulated with IL-17 (50 ng/ml) or TNF (10 ng/ml) for 4 h or left untreated (-) 20 h after transfection. Data are representative of three independent experiments. Graphs show mean ± SD, n = 3. *p<0.01.

Zhong et. al., Supplementary Figure 2



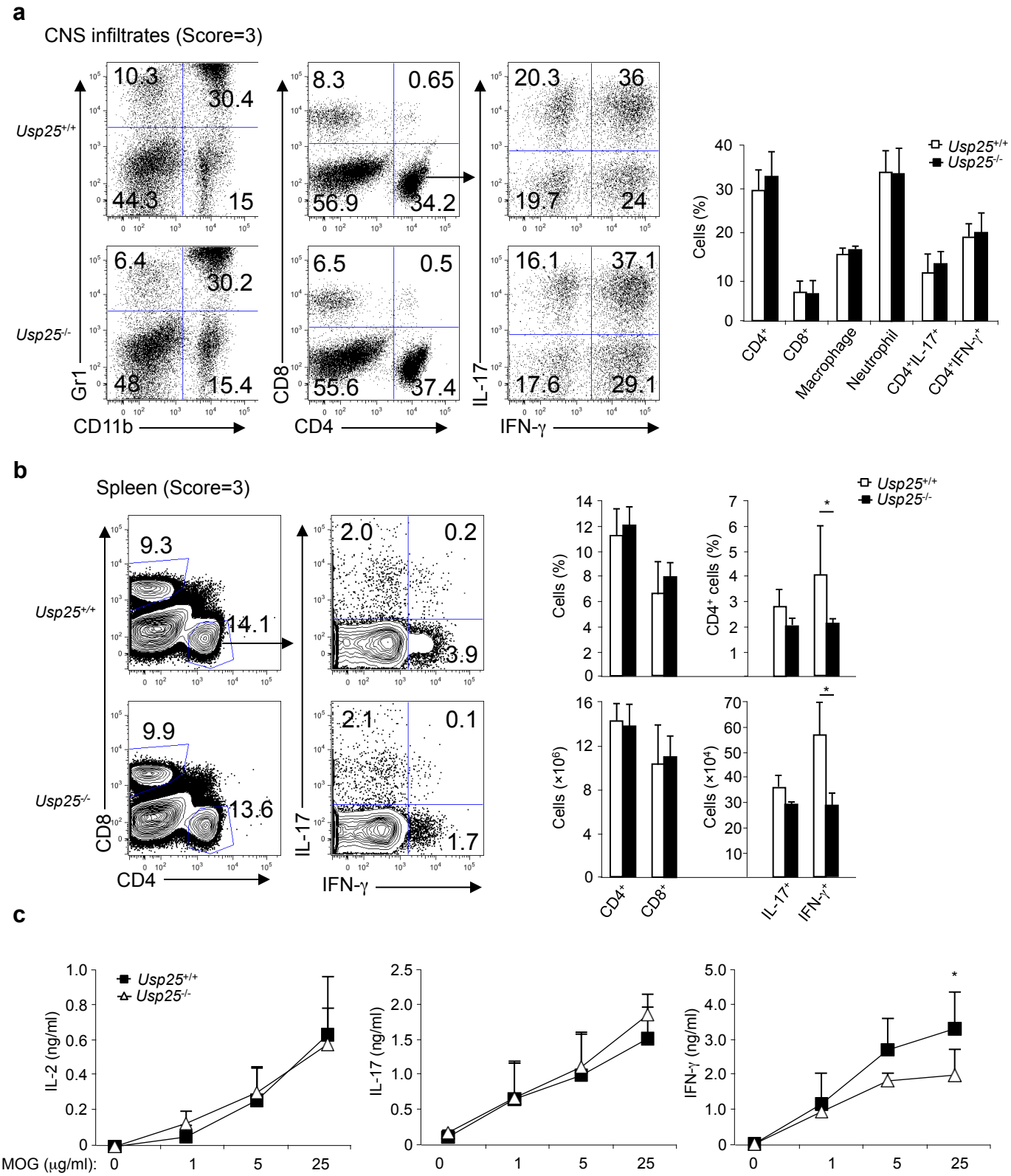
Supplementary Figure 2. Generation of *Usp25*$^{-/-}$ mice. (**a,b**), A schematic presentation of gene trapping strategy and genotyping results. SA, splicing adaptor sequence; β-gneo, β-galactosidase neomycin fusion gene; pA, polyA sequence; arrow head, genotyping primers. (**c,d**) Immonoblot of USP25 (**c**) or real-time PCR analysis of *Usp25* mRNA (**d**) in mouse embryonic fibroblasts (MEFs), spleen, thymus, lung or liver from wild-type and *USp25*$^{-/-}$ mice. For **c-d**, data are representative of two independent experiments. Graphs show mean ± SD, n=3.

Zhong et. al., Supplementary Figure 3



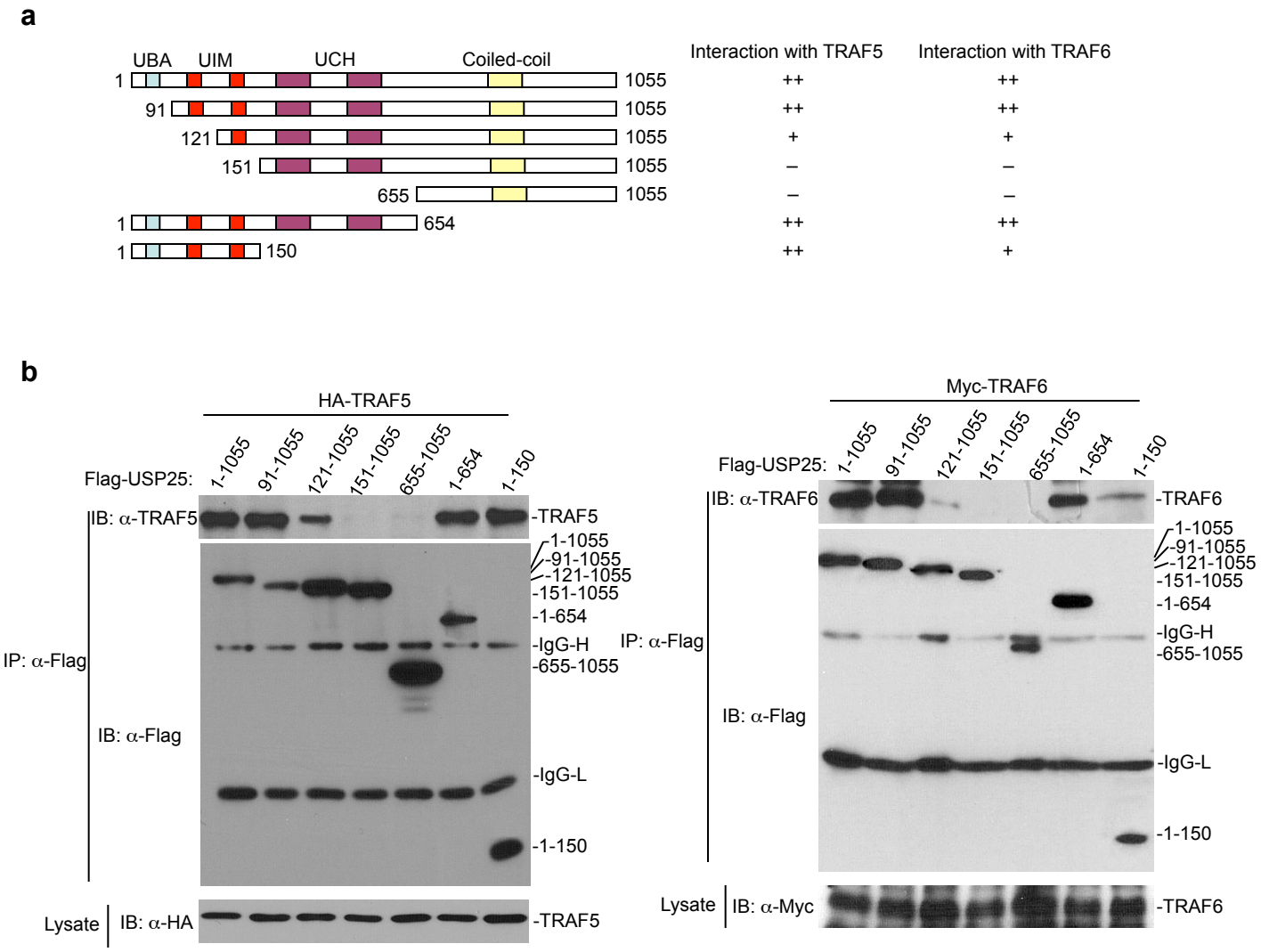
Supplementary Figure 3. USP25 deficiency has no effect on IL-1β-induced signaling. (**a,b**), Real-time PCR analysis of *Il6* mRNA (**a**) or ELISA analysis of IL-6 in the supernatants (**b**) of wild-type and *Usp25*$^{-/-}$ MEFs treated with IL-1β (10 ng/ml) for the indicated time points. (**c**) Immunoblot of lysates of wild-type and *Usp25*$^{-/-}$ MEFs treated with IL-1β (10 ng/ml) for the indicated time points with the indicated antibodies. Data are representative of three independent experiments. Graphs show mean ± SD, n = 3.

Zhong et. al., Supplementary Figure 4



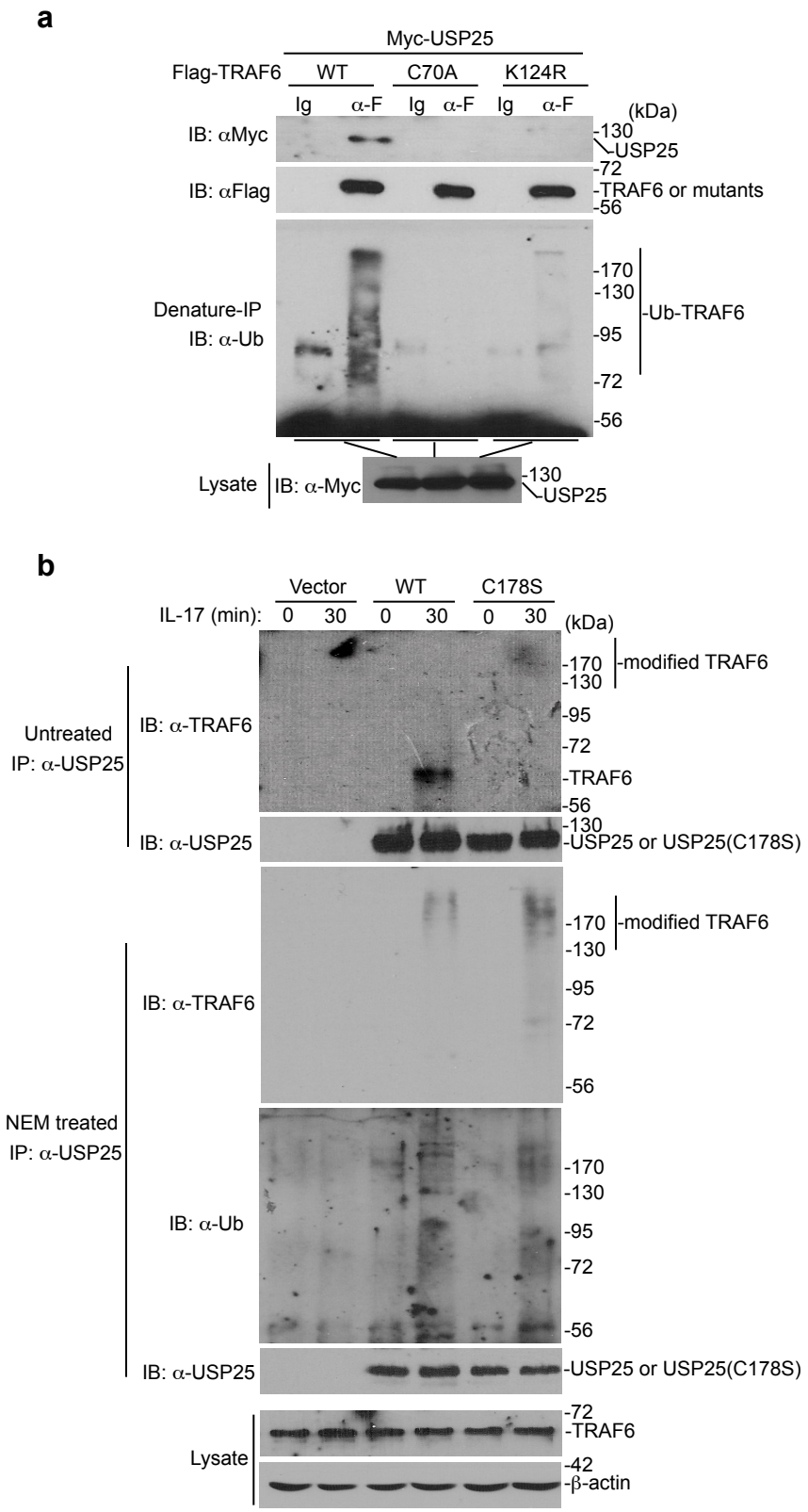
Supplementary Figure 5. Effects of USP25 deficiency on CNS infiltration and MOG-specific Th17 development during EAE induction. (**a**) Flow cytometry analysis of cell infiltrates in CNS system of wild-type or *Usp25*$^{-/-}$ mice induced EAE (score=3) (left panels). The percentages of different cell populations were statistically analyzed (right graph). (**b**) Flow cytometry analysisi of splenocytes from wild-type or *Usp25*$^{-/-}$ mice induced EAE (score=3) stimulated with MOG_{35-55} peptide (100 μg/ml) for overnight followed by treatment with golgi stop for 6 hours (left panels). The percentages and cell numbers of different cell populations were statistically analyzed (right graph). (**c**) ELISA analysis of IL-2, IL-17 and IFN-γ production by splenocytes from wild-type or *Usp25*$^{-/-}$ mice induced EAE stimulated with MOG_{35-55} peptide for three days. Graphs show mean ± SD, n = 5. Data are representative of three independent experiments.

Zhong et. al., Supplementary Figure 5



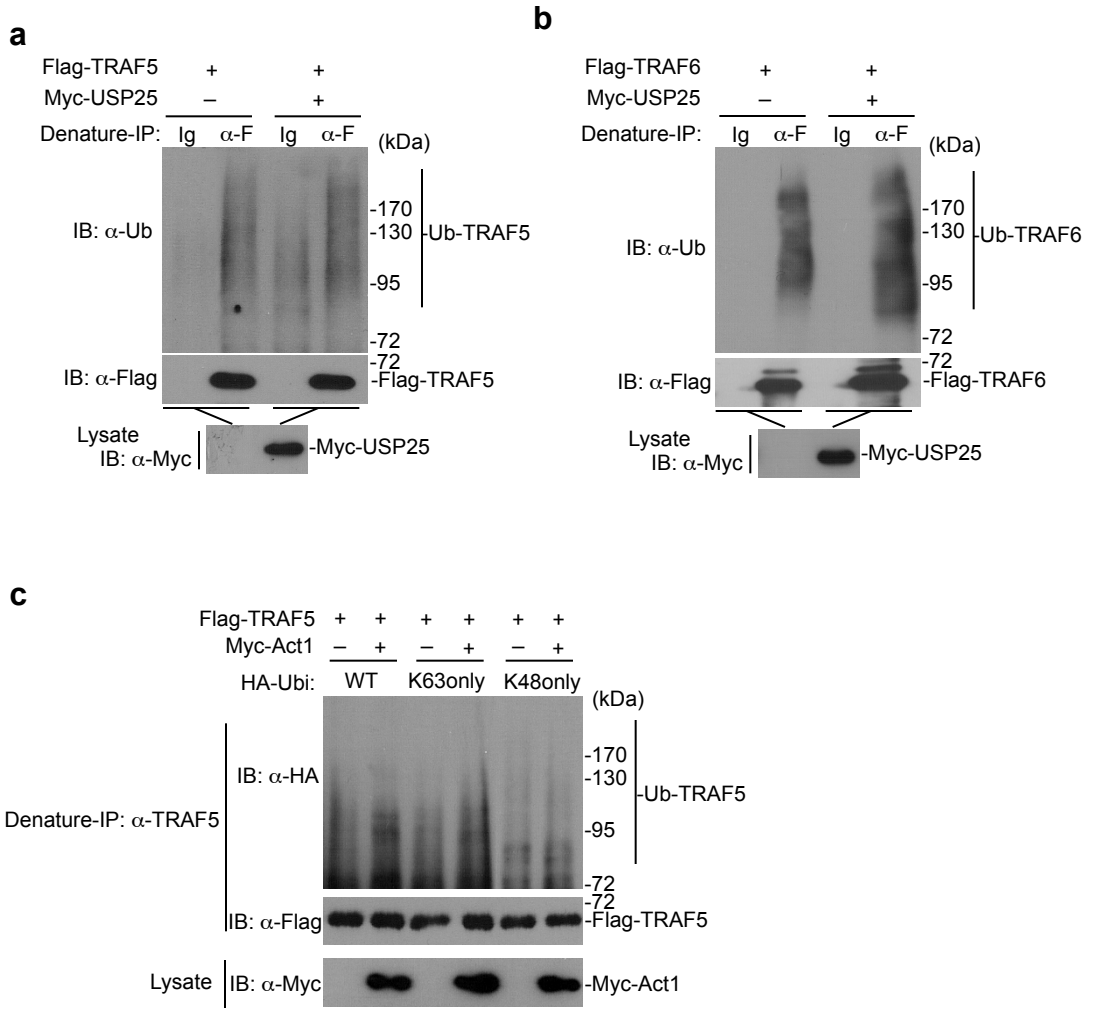
Supplementary Figure 5. Domain mapping for USP25-TRAF5 and USP25-TRAF6 interactions. (**a**) A schematic presentation shows USP25 domains and their ability to associate with TRAF5 or TRAF6. (**b**) The UBA-UIM of USP25 is required for optimal TRAF5-USP25 (left panels) and TRAF6-USP25 (right panels) interactions. HEK293T cells were transfected with the indicated plasmids. Immunoprecipitation assay was performed twenty hours after transfection. The immunoprecipitants were analyzed by immunoblot with anti-TRAF5 or anti-TRAF6 (top panels) or anti-Flag (middle panels). The expression levels of TRAF5 or TRAF6 were analyzed by immunoblot with anti-HA or anti-Myc (bottom panels). Data are representative of two independent experiments.

Zhong et. al., Supplementary Figure 6



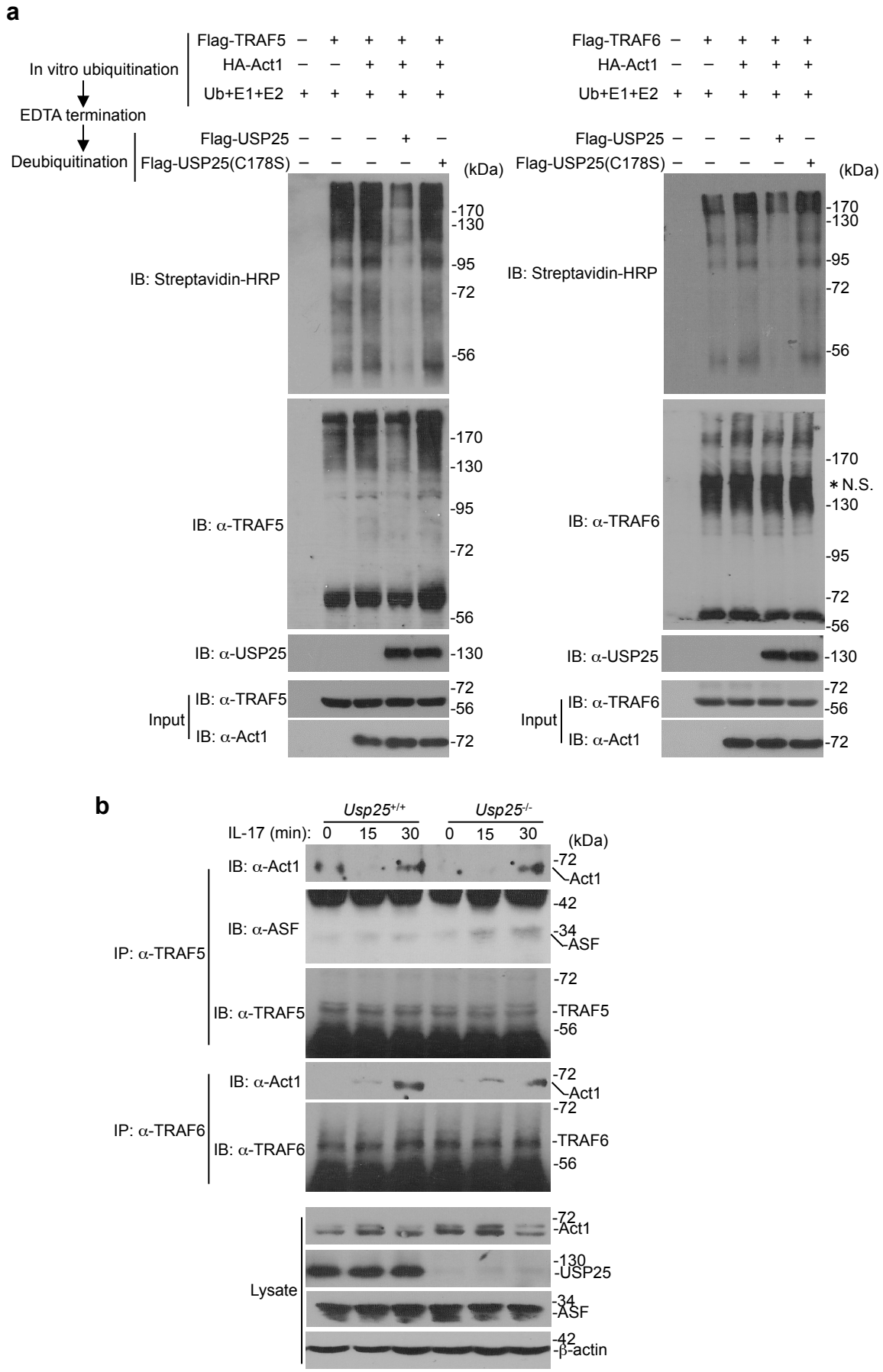
Supplementary Figure 6. TRAF6 modification mediates TRAF6-USP25 interaction. (**a**) Immunoblot assays of lysates from 293T cells transfected with the indicated plasmids, followed by immunoprecipitation with anti-Flag and immunoblot with anti-Myc (top two panels), or denature-IP with anti-Flag and immunoblot with anti-Ub (middle panel). The expression levels of Myc-USP25 in the lysates were determined by immunoblot with anti-Myc (bottom panel). (**b**) USP25(C178S) interacts with IL-17-induced modified TRAF6. USP25-deficient MEFs were reconstituted with empty vector, Flag-tagged USP25(WT) or USP25(C178S). Cells were treated with (middle and bottom panels) or without (top two panels) N-Ethylmaleimide (NEM, 0.5 mM) together with IL-17 (200 ng/ml) for 30 min. Cells were lysed and cell lysates were immunoprecipitated with anti-USP25 in the presence (middle and bottom panels) or absence (top two panels) of NEM (20 mM). The immunoprecipitants were analyzed by immunoblot with anti-TRAF6, anti-Ubiquitin or anti-USP25. The expression levels of TRAF6 in the lysates were analyzed by immunoblot with anti-TRAF6 or anti-β-Actin (bottom panels). Data are representative of two independent experiments.

Zhong et. al., Supplementary Figure 7



Supplementary Figure 7. Act1-mediated ubiquitination of TRAF5. (**a,b**) Immunoassay of 293T cells transfected with the indicated plasmids, followed by denature-immunoprecipitation (Denature-IP) with anti-Flag and immunoblot analysis with anti-ubiquitin (Ub) or anti-Flag 20 h after transfection. The expression levels of USP25 were analyzed by immunoblot with anti-Myc. (**c**) Immunoassay of 293T cells transfected with the indicated plasmids, followed by denature-immunoprecipitation (Denature-IP) with anti-TRAF5 and immunoblot analysis with anti-HA or anti-Flag 20 h after transfection. The expression levels of Act1 were analyzed by immunoblot with anti-Myc.

Zhong et. al., Supplementary Figure 8



Supplementary Figure 8. USP25 deubiquitinates Act1-mediated ubiquitination of TRAF5 and TRAF6 *in vitro*. (**a**) *In vitro* ubiquitination assays were carried out by mix of the indicated proteins before an aliquot of each mix was saved as input and analyzed with anti-TRAF5 or anti-TRAF6 or anti-Act1 (bottom two panels) , followed by addition of EDTA (10 mM) and USP25 or USP25(C178S). The reactions were subjected to immunobolt analysis with streptavidin-HRP (top panels) or anti-TRAF5 or anti-TRAF6 or anti-USP25 (middle panels). (**b**) Immunoaasays of lysates from wild-type and *Usp25*$^{-/-}$ MEFs treated with IL-17 for the indicated time points, followed by immunoprecipitation of anti-TRAF5 (top three panels) or anti-TRAF6 (middle two panels) and immunoblot with anti-Act1, anti-ASF, anti-TRAF5 or anti-TRAF6. Data are representative of two independent experiments.

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Genotyping primers for genomic DNA

WT forward	AATAGAAGTATGGGGGAATGGAA
WT reverse	CAAGAGGAGCACACAAGAACTTT
KO forward	CACCAACGTAACCTATCCCATTA
KO reverse	GGAAATCGCTGATTTGTGTAGTC

Genotyping primers for cDNA

WT forward	CCAACAAGCCCTGAAGGATA
WT reverse	GCCTGCTCTTCATCGGTTATGCC
KO forward	GCACCAGCAGACATTTTTGA
KO reverse	GACAGTATCGGCCTCAGGAAGATCG

Real-time PCR primers

Usp25 primer 1	TTGACCACCGGGAGAGCCGG
	TTCCAACAAGGGCCTGCCCA
Usp25 primer 2	TCCGGCACCAAGGCACATCAC
	ACGGCATGGAGGCGGTAAGG
Il6	TATGAAGTTCCTCTCTGCAAGAGA
	TAGGGAAGGCCGTGGTT
Tnf	AATGGCCTCCCTCTCATCAGT
	GCTACAGGCTTGTCACTCGAATT
Cxcl1	CGCTTCTCTGTGCAGCGCTGCTGCT
	AAGCCTCGCGACCATTCTTGAGTG
β-Actin	TGGAATCCTGTGGCATCCATGAAAC
	TAAAACGCAGCTCAGTAACAGTCCG
Human β-Actin	GGCCCGAGCCGGAGTAGCA
	GATGGACGGGAACACGGCCC
KcΔ4 RT	TTAATAAAACTTTTATTTT
Human Cxcl1	CTTCAGGAACAGCCACCAGT
	TCCTGCATCCCCCATAGTTA
Human Il6	AAACAACCTGAACCTTCCAAAGA
	GCAAGTCTCCTCATTGAATCCA